(1) Viral assembly is an intricate process involving protein folding, assembly and the interaction with nucleic acids. It is an important event in the life cycle of the virus and has received increasing attention over the past few years with an appreciation of the complexities of the problem. The great simplicity of the viruses, in comparison to cells makes them invaluable tools in the elucidation of gene structure and function, as well as the assembly of biological structures. In order to understand the molecular details of assembly one must also have an in-depth knowledge on the architecture of the viruses.

(2) The salient features on the architecture, assembly and disassembly of plant viruses is discussed in the **introductory chapter** of the thesis. In most instances, the assembly mechanisms *in vitro* are studied by reconstitution of isolated capsid protein with isolated nucleic acids. Such studies have lead to the identification of intermediates in the assembly of a helical virus such as tobacco mosaic virus, but in the case of icosahedral viruses, it is usually an all or none phenomenon. Further, in the case of viruses stabilized predominantly by protein-protein interactions, harsh conditions are required to disrupt the virions and these render the isolated protein assembly incompetent. Most recently, the mechanism of assembly of viruses is being unraveled employing capsid proteins and their mutants expressed in bacterial systems by using a combination of biophysical and crystallographic methods.

(3) The present thesis deals with the molecular details of assembly of physalis mottle tymovirus (PhMV), a member of tymovirus group, which is stabilized predominantly by protein-protein interactions. The current literature on tymoviruses in general and PhMV in particular has been reviewed in this chapter.

(4) PhMV consists of a monopartite single-stranded positive sense RNA genome encapsidated in an icosahedral shell of 180 identical subunits (subunit $\mathbf{M_r}$ 22 kDa). Purified preparations of PhMV contain two kinds of particles, the intact nucleocapsids and the empty capsids that can be separated by sucrose gradient centrifugation into denser bottom (PhMV-B) component and lighter top (PhMV-

T) component, respectively. *In vitro* assembly of any tymovirus from isolated components has not been reported so far.

(5) PhMV has been extensively studied in this laboratory. The complete coat protein sequence has been determined both by protein and nucleotide sequencing. UV-cross-linking studies showed that Lys 10 was involved in RNA-protein interaction and epitope mapping studies showed that the region 22-36 was exposed in the virus.

(6) The complete genome sequence of PhMV has been determined recently. The genome is 6.6 kb in length and has three open reading frames encompassing an overlapping protein, replicase protein and the coat protein.

(7) The aim of the current study was to resort to molecular biological tools to study the assembly process of PhMV. The objectives, therefore, were (a) to overexpress the coat protein gene of PhMV in *Escherichia coli* and to characterize the recombinant protein. (b) To determine the role of the amino and carboxy termini in capsid assembly and (c) identify residues involved in the intersubunit interactions.

(8) The general techniques used in the present investigation such as purification of the native, recombinant and mutant capsids, ELISA, western blotting, RNA slot-blot etc, with modifications if any from the published protocols, are described in **Chapter II** of the thesis.

(9) **Chapter III** describes the recloning of the coat-protein gene into other expression vectors and preliminary characterization of the overexpressed protein. The coat protein gene was cloned in pET 3d vector and overexpressed in Bl21(DE3) cells. The recombinant protein was found to self-assemble into capsids *in vivo*. The purified recombinant capsids had an apparent S value of 56.5 and a diameter of 29 ± 2 nm. These capsids were as stable as PhMV-T to high salt (2 M KCl), several cycles of freezing and thawing and to a wide range of pH (4.2-9.0). Urea denaturation studies on these capsids showed that the recombinant capsids were stable up to 4 M urea in contrast to PhMV-B, which was stable only up to 3 M urea. These capsids were also capable of encapsidating their own mRNA which was messenger active.

(10) In order to delineate further the role of the N and C- terminal residues in capsid assembly, six amino terminal deletion clones lacking the first 11, 26, 30,

34, 35 and 39 amino acid residues and 2 carboxy terminal deletions lacking the last 5 and 10 amino acid residues were constructed and overexpressed (**Chapter IV**). The proteins lacking N terminal 11 (PhCPN1), 26 (PhCPN2) and 30 (PhCPN3) amino acid residues self assembled into T=3 capsids *in vivo* as evident from electron microscopy, ultracentrifugation and agarose gel electrophoresis. The recombinant, PhCPN1, PhCPN2 and PhCPN3 capsids were as stable as the empty capsids formed *in vivo* and encapsidated a small amount of mRNA.

(11) The subtle conformational changes in the various deletion capsids were probed using the monoclonal antibody PA3B2. The monoclonal antibody PA3B2 that recognizes the epitope within region 22-36 failed to react with PhCPN2 capsids while it recognized the recombinant and PhCPN1 capsids. Thus, the epitope for the MAb mapped between the residues 22-26. Disassembly of the capsids upon treatment with urea showed that PhCPN2 capsids were the most stable.

(12) The deletions of 34, 35 and 39 amino acids rendered the protein insoluble. In the recently determined structure of PhMV, it is observed that the β-barrel begins at residue, 31 and deletions beyond 31 residues would perhaps interfere in the folding of the protein and lead to insoluble aggregates. These results demonstrate that N-terminal 30 amino acid residues are not essential for T=3 capsid assembly in PhMV. In contrast, both the proteins lacking the C-terminal 5 and 10 amino acid residues were present only in the insoluble fraction and could not assemble into capsids suggesting that these residues are crucial for folding and assembly of the particles.

(13) Assembly intermediates of icosahedral viruses are usually transient and are difficult to identify. In the **Chapter V,** site-specific and deletion mutants of the coat protein gene of PhMV were used to delineate the role of specific amino acids in the assembly of the virus and to identify intermediates in this process. Single C-terminal (N188) deletion mutant protein of PhMV and site-specific mutants H69A, C75A, W96A, D144N, D144N-T151A, K143E and N188A were constructed in pET 3d and expressed in BL21 (DE3) pLysS. The mutations were designed based on sequence comparison with other tymoviruses and on their uniqueness. All the site-specific mutants except N188A went into the insoluble fraction. Several methods of solubilization were tried but most of them were

unsucessful. Therefore, the mutants as well as the wild type coat protein was recloned into pRSET B vector.

(14) pRSET B vector has an advantage of the histidine tag that would help in purifying the proteins and it was hoped that the additional amino acids at the N-terminus would help in obtaining soluble proteins. Interestingly, the coat protein (pR PhCP) expressed using pRSET B vector with an additional 41 amino acids at the N-terminus also assembled into T=3 particles that were more compact and had a smaller diameter. These results demonstrate that the amino terminal segment is flexible and either the deletion or addition of amino acids at the N-terminus does not affect T=3 capsid assembly.

(15) In contrast, the deletion of even a single amino acid from the C-terminus (PhN188Δ1) resulted in capsids that were unstable. These capsids disassembled to a discrete intermediate with a sedimentation coefficient of 19.4 S. But the replacement of C-terminal asparagine 188 by alanine led to the formation of stable capsids.

(16) The C75A and D144N mutant proteins also assembled into capsids that were as stable as the pR PhCP suggesting that C75 and D144 are not crucial for the T=3 capsid assembly. pR PhW96A and pR PhD144N-T151A mutant proteins failed to form capsids and were present as heterogeneous aggregates.

(17) The pR PhK143E mutant protein behaved in a manner similar to the C-terminal deletion protein in forming unstable capsids. The intermediate with an *s* value of 19.4 was the major assembly product of pR PhH69A mutant protein and could correspond to a 30 mer. It is possible that the assembly or disassembly is arrested at a similar stage in pR PhN188Δ1, pR PhH69A and pR PhK143E mutant proteins. The data presented in this chapter are discussed in the light of the recently determined crystal structures of PhMV and TYMV.

(18) The results presented in this thesis demonstrate that the N-terminal arm, shown to be ordered in all the subunits of PhMV in the crystal structure, is flexible and can assume different conformations in solution. Stable T=3 capsids can be formed upon either deletion of 30 amino acids or addition of 41 amino acids at the N-terminus. The C-terminal asn, involved in extensive quasi 2-fold and quasi 3-fold contacts, is crucial to the assembly of the virus. For the first time a sub assembly intermediate was identified and characterized in the present study.